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HrcQ is necessary for *Xanthomonas oryzae* pv. *oryzae* HR-induction in non-host tobacco and pathogenicity in host rice

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ABSTRACT

Bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most destructive diseases of rice (*Oryza sativa* L.) worldwide. The type III secretion system (T3SS) of *Xoo*, encoded by the *hrp* (hypersensitive response and pathogenicity) genes, plays critical roles in conferring pathogenicity in host rice and triggering a hypersensitive response (HR) in non-host plants. To investigate the major genes conferring the pathogenicity and avirulence of *Xoo*, we previously constructed a random Tn5-insertion mutant library of *Xoo* strain PXO99^{A}. We report here the isolation and characterization of a Tn5-insertion mutant PXM69. Tn5-insertion mutants were screened on *indica* rice JG30, which is highly susceptible to PXO99^{A}, by leaf-cutting inoculation. Four mutants with reduced virulence were obtained after two rounds of screening. Among them, the mutant PXM69 had completely lost virulence to the rice host and ability to elicit HR in non-host tobacco. Southern blotting analysis showed a single copy of a Tn5-insertion in the genome of PXM69. PCR walking and sequencing analysis revealed that the Tn5 transposon was inserted at nucleotide position 70,192–70,201 in the genome of PXO99^{A}, disrupting the type III *hrc* (*hrp*-conserved) gene *hrcQ*, the first gene in the D operon of the *hrp* cluster in *Xoo*. To confirm the relationship between the Tn5-insertion and the avirulence phenotype of PXM69, we used the marker exchange mutagenesis to create a PXO99^{A} mutant, Δ*hrcQ*::KAN, in which the *hrcQ* was disrupted by a kanamycin-encoding gene cassette at the same site as that of the Tn5-insertion. Δ*hrcQ*::KAN showed the same phenotype as mutant PXM69. Reintroduction of the wild-type *hrcQ* gene partially complemented the pathogenic function of PXM69. RT-PCR and cellulase secretion assays showed that the Tn5-disruption of *hrcQ* did not affect transcription of downstream genes in the D operon and function of the type II secretion system. Our results provide new insights into the pathogenic functions of clustered *hrp* genes in *Xoo*.

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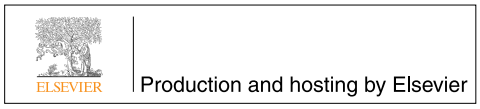

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1. Introduction

Bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most devastating bacterial diseases of rice (*Oryza sativa* L.) [1]. *Xoo* invades rice plants through water pores and wounds on leaves, causes a vascular disease and manifests by tannish-gray to white lesions along the leaf veins [2]. *Xoo*, like many other Gram-negative plant-pathogenic bacteria, relies on the type III secretion system (T3SS) to inject effector proteins into host cells, leading to either disease or a resistance reaction [3]. T3SS of *Xoo*, encoded by the *hrp* (*h*ypersensitive response and *p*athogenicity) genes, is an essential determinant of bacterial pathogenicity, which is achieved by controlling the secretion and translocation of effector proteins that cause disease in susceptible hosts [4]. In resistant host and non-host plants, T3SS is involved in the induction of a hypersensitive response (HR), a local programmed cell death that inhibits pathogen multiplication at the infection site [5,6].

The *hrp* genes of *Xanthomonas* are highly conserved and clustered [7], comprising of nine *hrp* genes, nine *hrc* (*hrp*-conserved) genes, and eight *hpa* (*hrp*-associated) genes in *Xoo* [8]. It is generally accepted that the expressions of *hrp* genes are controlled by HrpG and HrpX [9]. Recently, Li et al. [10] demonstrated that, apart from HrpG and HrpX, HrpD6 also plays an important role in the regulation of *hrp* genes in *X. oryzae* pv. *oryzicola* (*Xoc*). Another study showed that there were three transcription units within the core region of the *hrp* gene cluster in *Xoo*, namely *hrpB1* to *hrcC*, *hrcU* to *hpaP* and *hrcQ* to *hpaB* [9]; disruption of the *hrcQ* gene in *Xoo* caused the loss of the pathogenicity to rice, but it was unclear if the loss of pathogenicity could be restored by the wild-type *hrcQ*.

In a previous study, we constructed a Tn5-tagged PXO99^A mutant library, consisting of 24,192 *Xoo* transformants (clones), with a six times coverage of the PXO99^A genome [11]. In an attempt to identify major virulence genes in PXO99^A, we screened the library and isolated mutants with reduced virulence. Here, we reported the isolation and characterization of a *hrcQ*-Tn5-insertion mutant PXM69 with no virulence in host rice and no ability to elicit HR in non-host tobacco (*Nicotiana benthamiana*). We found that reintroduction of the *hrcQ* gene could only partially complement the loss of pathogenic function in PXM69.

2. Materials and methods

2.1. Bacterial strains and plant materials

Xoo strains used in this study were PXO99^A (wild-type) and its mutants such as PXM69. *Escherichia coli* strain DH5α was used in constructing plasmids for marker exchange mutagenesis. *Xoo* strains were grown at 28 °C on TSA medium (tryptone, 10 g L^{-1}; sucrose, 10 g L^{-1}; glutamic acid, 1 g L^{-1}; and agar, 15 g L^{-1}; pH 6.8–7.0) or NB medium (peptone, 5 g L^{-1}; yeast extract, 1 g L^{-1}; sucrose, 10 g L^{-1}; and beef extract, 3 g L^{-1}; pH 6.8–7.0). The *E. coli* strain was grown at 37 °C in Lutia–Bertani medium (tryptone, 10 g L^{-1}; yeast extract, 5 g L^{-1}; and sodium chloride, 10 g L^{-1}; pH 6.8–7.0). The broad host-range vector pHM1 was used to produce complementary constructs. Antibiotics used in the study were ampicillin (Amp) 100 μg mL^{-1}, kanamycin (Km) 50 μg mL^{-1}, spectinomycin (Sp) 100 μg mL^{-1}, and rifampicin (Rf) 50 μg mL^{-1}.

The *indica* rice cultivar JG30, highly susceptible to PXO99^A, was planted in the field or in a greenhouse reaching 28–32 °C in daylight hours. Inoculations were performed on the plants at the maximum tillering stage (40 to 50 days old) by the leaf-clipping method [12]. *N. benthamiana* plants were grown in a growth cabinet under standard conditions (day and night temperatures of 25 °C and 20 °C, respectively), with 16 h light (30 to 40 μmol s^{-1} m^{-2}) and 50%–60% humidity. Expanded leaves of 5- to 7-week-old plants were inoculated using a needleless syringe [10].

2.2. Assays of virulence and hypersensitive response and measurement of bacterial growth in rice

The pathogenicity of all *Xoo* strains was evaluated using the leaf-cutting method [12]. In the first round of screening for Tn5-insertion mutants, saturated cultures of *Xoo* strains grown in TSA medium were pelleted down and re-suspended in sterile distilled water (SDW) at an optical density of 1.0 at 600 nm (OD_{600} 1.0). Scissors dipped in the inocula were used to clip fully expanded leaves of JG30. Disease symptoms were recorded two weeks after inoculation. To isolate mutant candidates with reduced virulence, leaves with lesions shorter than 2 cm were collected and clipped into several pieces of 2 cm × 1 cm, soaked in 70% ethanol for 30 s, washed with SDW for 10 s, and then soaked in 1 ml SDW for about 20 min. The solution was spread on tryptone sucrose medium containing at 50 μg mL^{-1} of kanamycin. In the second round of screening, each of the reduced virulence mutant candidates was inoculated to three JG30 plants. For each plant, at least three fully expanded leaves were inoculated. Two weeks after inoculation, the lesion lengths on the inoculated leaves were measured. Disease symptoms were scored as lesion length.

Xoo strains were cultured on TSA plates with appropriate antibiotics, pelleted down, re-suspended in SDW at OD_{600} 0.5, and then individually infiltrated into leaves of *N. benthamiana* with needleless syringes. At 36 to 72 h post-infiltration, HR triggered by *Xoo* in the form of necrotic regions at the area of inoculation was recorded. The experiments were repeated three times.

Xoo strains were incubated in PSA medium (polypeptone, 10 g L^{-1}; sucrose, 10 g L^{-1}; and glutamic acid, 1 g L^{-1}; pH 6.8–7.0) and shaken at 250 r min^{-1} and 28 °C for 42 h. Bacterial suspensions were adjusted to a concentration of about 1 × 10^9 CFU mL^{-1} (OD_{600} 1.0) with SDW and infiltrated into fully expanded leaves of 4-week-old JG30 plants with needleless syringes. For each strain, three plants were inoculated, and three 1-cm^2 leaf disks from different infiltrated leaves were harvested as one sample. After sterilization in 70% ethanol, the disks were ground in a sterilized mortar with a pestle in 4 mL SDW, and plated at different concentrations to determine the CFU cm^{-2}. Serial dilutions were spotted in triplicate onto TSA plates with appropriate antibiotics. The plates were incubated at 28 °C for 3 to 4 days until colonies could be counted. The experiments were repeated three times.

2.3. *Molecular characterization of the Tn5-insertion mutant*

Total genomic DNA of PXO99^{A} and its mutant were isolated as described by Leach et al. [13]. The polymerase chain reaction (PCR) was performed to check the inserted Tn5-DNA fragment using primers Tn5F and Tn5R (Table 1), and the expected PCR product was 569 bp in length. The solution (20 μL) contained 50 ng of template DNA, 1× PCR buffer, 0.3 mmol L^{-1} dNTPs, 0.3 μmol L^{-1} each primer, and 1.0 U KOD *Taq* polymerase (TOYOBO, Japan). PCR was initiated at 95 °C for 3 min followed by 34 cycles of amplification at 94 °C for 40 s, 60 °C for 40 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min.

For Southern blotting, genomic DNA of *Xoo* strains was digested with *Sph*I (TaKaRa), separated on 1.2% (W/V) agarose gel by electrophoresis, alkali-denatured and transferred onto Hybond-N^{+} membranes. The DNA probe was amplified from an EZ-Tn5 <KAN-2> Tnp Transposome DNA template by PCR using the primers Tn5F and Tn5R. The probe was labeled with [α–^{32}P] dCTP using Random Primer DNA Labeling Kit (TaKaRa) according to the manufacturer's instruction. Prehybridization, hybridization, and posthybridization washes were carried out as described by Wang et al. [11]. Blots were exposed on a PhosphorImager plate and signals were detected by Molecular imager FX (Bio-Rad).

Genomic DNA fragments flanking the Tn5-insertion site in the mutant were amplified by PCR-walking [14]. Tn5-insertion mutant DNA was digested by *Eco*R V (TaKaRa) and ligated with the designed adaptor [11]. The adaptor specific primers AP1, AP2, and Tn5-specific primers TnFP1, TnRP1, TnFP2 and TnRP2 were designed for isolating the forward and reverse flanking sequences (Fig. 3-a). PCR products were retrieved and purified for sequencing. By aligning both the forward and reverse flanking sequences with the whole genome sequences of *Xoo* strains PXO99^{A}, KACC10331 and MAFF311018 through NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST), the Tn5-insertion site in the mutant was determined.

2.4. *Marker exchange mutagenesis*

Marker exchange was performed by splice overlap PCR. A fragment containing a kanamycin-encoding gene cassette (KM) was amplified from pKD13 plasmid DNA using primers KD13F and KD13R (Table 1). The *hrcQ* forward flanking fragment (hrcQF1R1) and reverse flanking fragment (hrcQF2R2) were amplified from PXO99^{A} genomic DNA using the primer pairs P69F1/P69R1 and P69F2/P69R2, respectively. Primer P69R1 contains the forward flanking sequence of KM, and P69F2 contains the reverse flanking sequence. The hrcQF1R1 and KM fragments were mixed as template, and primers P69F1 and KD13R were used to amplify the forward fragment hrcQF-KM. Similarly, the reverse fragment KM-hrcQR was amplified with primers KD13F and P69R2. The hrcQF-KM and KM-hrcQR fragments were individually ligated into the pBluescript II SK (–) vector at an *Eco*R V restriction enzyme site. The *Eco*R I site (in the SK vector) and *Nco* I site (in the kanamycin-encoding gene cassette) were used to construct the plasmid SK-hrcQ. After confirming the insertion by DNA sequencing, the SK-hrcQ plasmid was

Table 1 – Primers used in this study.

Primer	Sequence (5′–3′)	Description
Tn5F	ATTCAACGGGAAACGTCTTG	For the 569 bp Tn5-DNA fragment
Tn5R	ACTGAATCCGGTGAGAATGG	
AP1	GGATCCTAATACGACTCACTATAGGGC	Adaptor specific primer
AP2	CTATAGGGCTCGAGCGGC	Adaptor specific primer
TnFP1	GGCAGAGCATTACGCTGACT	Tn5 specific primer
TnFP2	ACCTACAACAAAGCTCTCATCAACC	Tn5 specific primer
TnRP1	CTGATTGCCCGACATTATCG	Tn5 specific primer
TnRP2	GCAATGTAACATCAGAGATTTTGAG	Tn5 specific primer
PXM69F7	CAAAGCTTCGACACCAGCAATGCAACGCCATTGAGGAC	For the 1326 bp fragment of *hrcQ* gene with
PXM69R5	GTGAATTCAGTTCGCCAGACGCGCGCGAGGTACTTTGG	its own promoter
KD13F	ACGTCTTGAGCGATTGTGTAGGCT	For the 1408 bp fragment of the
KD13R	AGTGATTGCGCCTACCCGGATATT	kanamycin-encoding gene cassette
P69F1	CAGGCATCTGCATGCGTGCTCTC	For the 485 bp forward flanking fragment (hrcQF1R1)
P69R1	AGCCTACACAATCGCTCAAGACGTGCACCGCATCAGCTGC	
P69F2	AATATCCGGGTAGGCGCAATCACTGCCCGCCGATGCGCAG	For the 385 bp reverse flanking fragment (hrcQF2R2)
P69R2	GTGGATACCGGCCAGATGGATTTG	
RT-hrcQF	CAGAATCATCTGTTGCATATTC	For the 360 bp fragment of *hrcQ*
RT-hrcQR	GCCTTCAAGCGCGATGCAAC	
RT-hrcRF	GTCCGACTTGAGCGTGGTGG	For the 404 bp fragment of *hrcR*
RT-hrcRR	ATGCAGATGCCTGACGTTGG	
RT-hrcSF	TCATGGGAACGCCGCCTGCATC	For the 204 bp fragment of *hrcS*
RT-hrcSR	GTGTCCTTGCCGGTGGTCGG	
RT-hpaAF	CGAAACGCCGATGGCGCGCAG	For the 360 bp fragment of *hpaA*
RT-hpaAR	CGACGACGCGGGAACCGGTC	
RT-hrpD6F	TTACCGCATATTTGCGATATG	For the 243 bp fragment of *hrpD6*
RT-hrpD6R	ATGTTCGATGCAATGACCG	
RT-hrpEF	TCACTGGCCAACGAGCTGCT	For the 159 bp fragment of *hrpE*
RT-hrpER	ATGGGTTCGTTGCTCGGCGA	
rRNA-F	TAGCTCAGGTGGTTAGAGCGC	For the 240 bp fragment of *16SrRNA*
rRNA-R	CAACGCGAACATACGACTCAA	

transferred into a wild-type strain PXO99^A by electroporation. The cell cultures were spread on TSA medium plates containing kanamycin (Km) at 50 μg mL^{-1}, incubated at 28 °C for 3 to 4 days. Clones were picked out and cultured in TSA medium plates containing ampicillin (Amp) at 100 μg mL^{-1} for the second selection. We picked clones that grew on the kanamycin-containing plates but not on the ampicillin-containing plates.

2.5. *Functional complementation of the Tn5-insertion mutant*

According to the Tn5-insertion site and genome sequence of PXO99^A, the wild-type *hrcQ* gene with its promoter was amplified by PCR using primers PXM69F7 and PXM69R5 (Table 1). The PCR product, with *Hin*d III and *Eco*R I restriction sites introduced at the two ends, respectively, was cloned into the pEASY-B (TransGen) vector. After the DNA insert was confirmed by sequencing, the *hrcQ*-containing fragment was cut out by *Hin*d III and *Eco*R I digestion and cloned into the broad host range vector pHM1, resulting in the complementary plasmid pHhrcQ, which was then transferred into the mutant strain PXM69 by electroporation using a Gene Pulser Xcell (Bio-Rad) electroporator at 1.8 kV mm^{-1}. After electro-pulsing cells were incubated in 500-μL PSA medium in a 200 r min^{-1} rotary shaker at 28 °C for 1.5 h. Following recovery cell cultures were spread on TSA medium plates containing spectinomycin (Sp) at 100 μg mL^{-1}, and then the plates were incubated at 28 °C for 3 to 4 days. Clones were picked out and cultured in PSA medium for virulence assays in rice and tobacco.

2.6. *RT-PCR analysis*

Xoo strains were inoculated into 20 mL of PSA medium and grown at 28 °C for 24 to 36 h until an optical density of 0.8 at 600 nm (OD_{600}) reached. This culture (2 mL) was transferred into 50 mL of fresh PSA and incubated for another 12 to 16 h until the OD_{600} reached 0.6. After centrifugation at 6000 r min^{-1} for 10 min at 4 °C, the cell pellet from 15 mL of bacterial culture was twice washed in sterilized water. The cell pellet was re-suspended in 15 mL of hrp-inducing medium XOM3 (pH 6.5) [10] at 28 °C for 16 h. Bacteria were collected by centrifugation at 12,000 r min^{-1} for 2 min and total RNA was extracted using a TRIzol kit (Invitrogen). The extracted RNA was purified with an RNAprep Pure Cell/Bacteria kit (Tiangen), and then used as template for PCR amplification of *hapD6* to ensure that the RNA samples contained no contamination with genomic DNA. Total RNA (1 μg) was used to synthesize cDNA using a FastQuant RT kit (Tiangen) with random primers. The reaction was performed at 42 °C for 8 min, 42 °C for 1 h, and inactivated at 95 °C for 3 min. The cDNA product (1 μL) and gene-specific primers (Table 1) were used in RT-PCR with the following program: step 1, 94 °C for 3 min; step 2, 94 °C for 40 s; step 3, 58 °C for 40 s; step 4, 72 °C for 60 s; then 35 cycles (unless specifically indicated) repeating from steps 2 to 4; and finally step 5, 72 °C for 10 min.

2.7. *Cellulase secretion assay*

Xoo strains were cultured up to OD_{600} 1.0 in PSA medium with appropriate antibiotics in a 230 r min^{-1} rotary shaker at 28 °C. Cells from 1 mL of culture were harvested by centrifugation at 6000 r min^{-1} for 2 min at 4 °C, twice washed with SDW, and re-suspended with SDW to 1 mL. The suspended cells were spot inoculated in the CMC selection medium (NaCl, 6.0 g L^{-1}; $MgSO_4$, 0.1 g L^{-1}; KH_2PO_4, 0.5 g L^{-1}; $CaCl_2$, 0.1 g L^{-1}; $(NH_4)_2SO_4$, 2.0 g L^{-1}; K_2HPO_4, 2.0 g L^{-1}; CMC-Na, 5.0 g L^{-1}; yeast, 1.0 g L^{-1}; and agar, 15 g L^{-1}; pH 7.0) at 28 °C for 48 h. Secretion of cellulase was detected by formation of transparent halos against the red background after staining with 0.1% Congo Red and washing with 1 mol L^{-1} NaCl solution.

3. Results

3.1. *Tn5-insertion mutant PXM69 lacks both pathogenicity in the rice host and HR-triggering ability in non-host tobacco*

A total of 15,440 clones of the Tn5-PXO99^A mutant library were screened in the first round of inoculation, and seven mutants (clones) displayed reduced virulence phenotypes in the rice variety JG30. To confirm reduced virulence, we isolated these mutants from infected leaves and conducted a second round of screening. Finally, four mutants with stable reduced pathogenicity in JG30 were identified, and designated PXM36, PXM37, PXM69 and PXM73. Among them, mutant PXM69 with complete loss of pathogenicity in JG30 (Fig. 1-a, b) was chosen for extensive investigation. The bacterial growth rate of PXM69 in rice tissue was significantly reduced compared to wild-type strain PXO99^A (Fig. 1-c). We then infiltrated PXM69 cells into tobacco leaves to assess their ability to elicit HR in non-host plants. PXM69 had also lost its ability to induce HR in tobacco (Fig. 1-d).

3.2. *A single Tn5-insertion in the hrcQ gene is present in mutant PXM69*

The mutant PXM69 was first analyzed by PCR using primers Tn5F and Tn5R (Table 1). An expected 569 bp DNA fragment was amplified from the genomic DNA of PXM69 (Fig. 2-a), confirming the presence of a Tn5-insertion in the genome. In order to determine the copy number of the Tn5-insertion in the genome of PXM69, genomic Southern blotting analysis was conducted. The genomic DNA was digested with *Sph* I, and a single hybridization band was detected by the Tn5-derived probe, whereas the wild-type PXO99^A displayed no hybridization band (Fig. 2-b), indicating that there was a single Tn5-insertion in the genome of the mutant PXM69.

PCR walking [14] was then used to isolate the flanking sequences of the Tn5-insertion site in PXM69. Nested PCR with primer pairs Ap1/TnRP1 and Ap2/TnRP2 was performed to isolate the left flanking sequences (Fig. 3-a). Similarly, nested PCR with primer pairs Ap1/TnFP1 and Ap2/TnFP2 was performed to isolate the right flanking sequences (Fig. 3-a). The nested PCR products were sequenced and compared with the genome sequences of *Xoo* PXO99^A, KACC10331 and MAFF311018 by NCBI BLASTN and BLASTX searches. As shown in Fig. 3-b, the Tn5 transposon was inserted at nucleotide position 70192/201 in the genome of PXO99^A, disrupting the type III *hrc* (*hrp*-conserved) gene *hrcQ*, the first gene in the D operon of the *hrp* gene cluster [9].

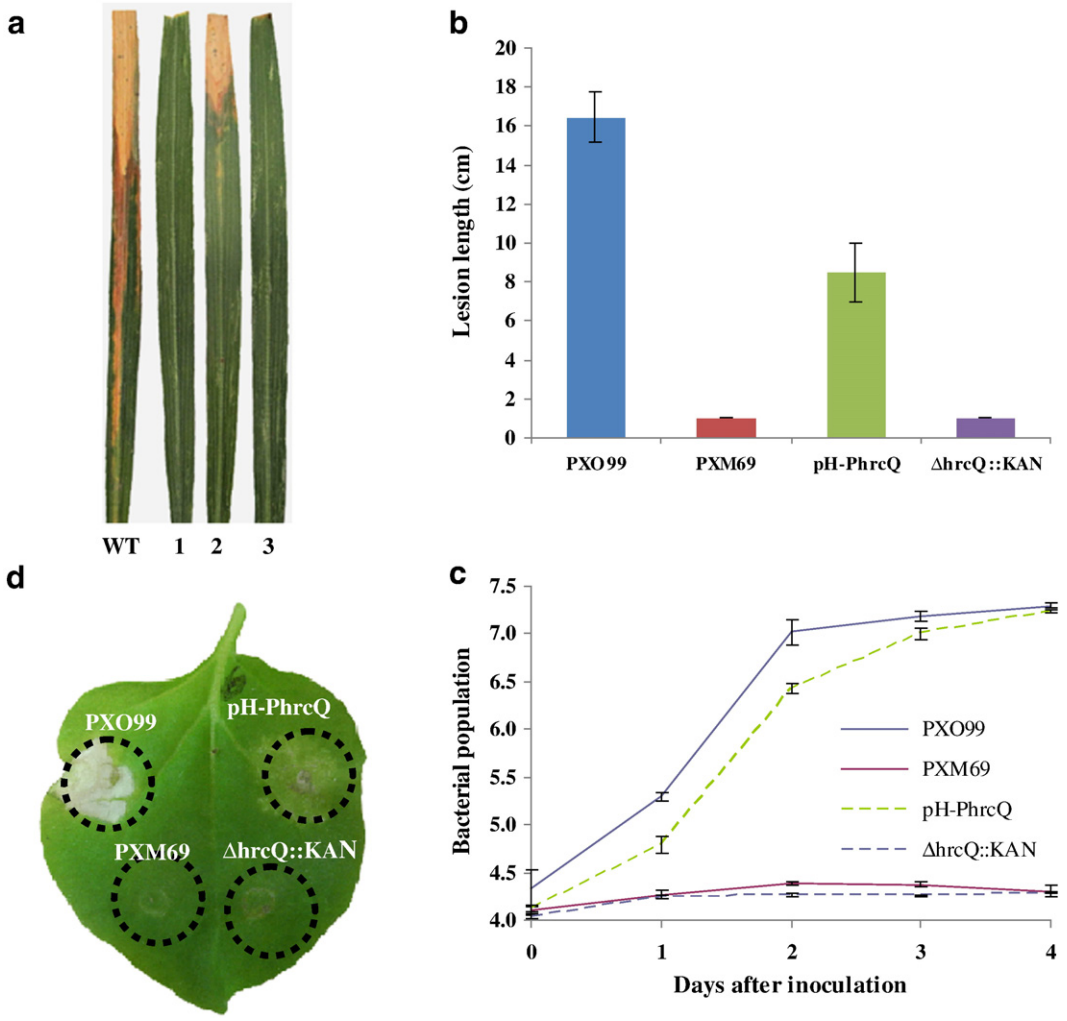

Fig. 1 – Pathogenicity assays with Tn5-insertion mutant PXM69. a and b: Virulence assay in rice JG30 by artificial leaf-cutting inoculation. Pictures were taken and lesion lengths were measured 14 days after inoculation; c: Bacterial population [log·(CFU cm^{-2})] in JG30. Bacteria were recovered from inoculated leaves each day for a period of 4 days after inoculation. Means and standard deviations were from 3 replicate experiments; d: HR assay in non-host plant *N. benthamiana* leaves by infiltration with *Xoo* cells adjusted to OD_{600} 0.5 using a needleless syringe; HR was triggered by strains $PXO99^A$ and pH-PhrcQ 2–4 days after infiltration, but not by mutant PXM69 or Δ*hrcQ*::KAN.

3.3. *hrcQ-Disruption mutant ΔhrcQ::KAN confirmed the loss of virulence in PXM69*

To confirm whether the loss of pathogenicity in PXM69 was caused by Tn5-disruption of the *hrcQ* gene, we recreated a disruption mutant Δ*hrcQ*::KAN of $PXO99^A$ by marker exchange mutagenesis at the same site as that of Tn5-insertion in PXM69. As expected, pathogenicity assays showed that Δ*hrcQ*::KAN also lost the virulence on JG30 and the ability to induce HR in non-host tobacco (Fig. 1-a, d). The growth of Δ*hrcQ*::KAN in rice tissue was also significantly inhibited compared to wild-type $PXO99^A$ (Fig. 1-c).

3.4. *Pathogenicity and HR-triggering ability of PXM69 were partially restored by ectopic expression of hrcQ*

The *hrcQ* gene with its promoter region (1326 bp: 69,569–70,894 in GenBank accession no. CP000967.1) was amplified by PCR and cloned into the broad host range plasmid pHM1, resulting in plasmid pHhrcQ, which was then transferred into the Tn5-insertion mutant PXM69 by electroporation, and the complementary strain pH-PhrcQ was obtained. Pathogenicity assays were performed using the leaf-clipping method. Results showed that bacterial growth of pH-PhrcQ in rice tissue was almost fully restored (Fig. 1-c). However, the lesion length caused by pH-PhrcQ was not as long as that by the wild-type strain $PXO99^A$, indicating that the pathogenicity was not completely recovered, although the pH-PhrcQ caused much longer lesions than PXM69 (Fig. 1-a). HR assay results also indicated that pH-PhrcQ partially recovered the ability of HR-triggering (Fig. 1-d).

3.5. *Expression of genes in the D operon in PXM69*

Since the pathogenicity and the HR-triggering ability of PXM69 could only be partially complemented by reintroduction of the

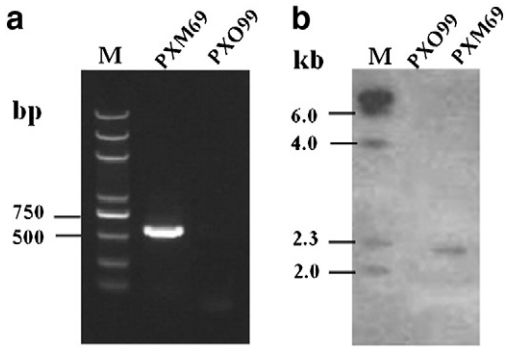

Fig. 2 – Molecular characterization of mutant PXM69. a: Identification of the Tn5-DNA inserted in the genome of the mutant by PCR using primers Tn5F and Tn5R; the expected PCR product is 569 bp. b: Southern blotting analysis to determine the copy number of Tn5-insertions in the mutant. A single copy of Tn5-insertion was detected in PXM69.

hrcQ gene, we speculate that expression of the introduced *hrcQ* gene, or other genes in the D operon, was affected. Thus semiquantitative RT-PCR was performed.

As shown in Fig. 4, the expression of *hrcQ* was completely abolished by the Tn5-insertion in mutant PXM69, whereas the introduced *hrcQ* was highly expressed in the complementary strain pH-PhrcQ. Expressions of the downstream genes *hrcR*, *hrcS*, *hpaA*, *hrpD6* and *hrpE* were similar to those of the wild-type strain PXO99^{A}, indicating that the Tn5-insertion in *hrcQ* is non-polar. We also conducted RT-PCR of *hrpD6* and *hpaA* and found that their expressions were very low and again with no differences from those of the wild-type strain PXO99^{A} (data not shown). These results indicate that the partial complementation of the pathogenicity of PXM69 was not due to the expression of *hrcQ* or the downstream genes in the D operon at transcriptional level.

3.6. *hrcQ-Disruption did not affect the function of the type II secretion system*

Plant pathogenic bacteria produce numerous extracellular enzymes to degrade host cell walls. The extracellular enzymes such as cellulase are secreted by a type II secretion system [15]. Because the *hrcQ*-disrupted mutants had completely lost their virulence, and the complementary strain could not fully restore its pathogenicity, we sought to investigate whether the function of the type II secretion system in PXM69 was also affected by assaying cellulase secretion. As shown in Fig. 5, the transparent halos of the mutants and complementary strain were similar in size and were no different from wild-type PXO99^{A}. Thus, *hrcQ*-disruption did not affect the function of the type II secretion system of *Xoo* mutant PXM69.

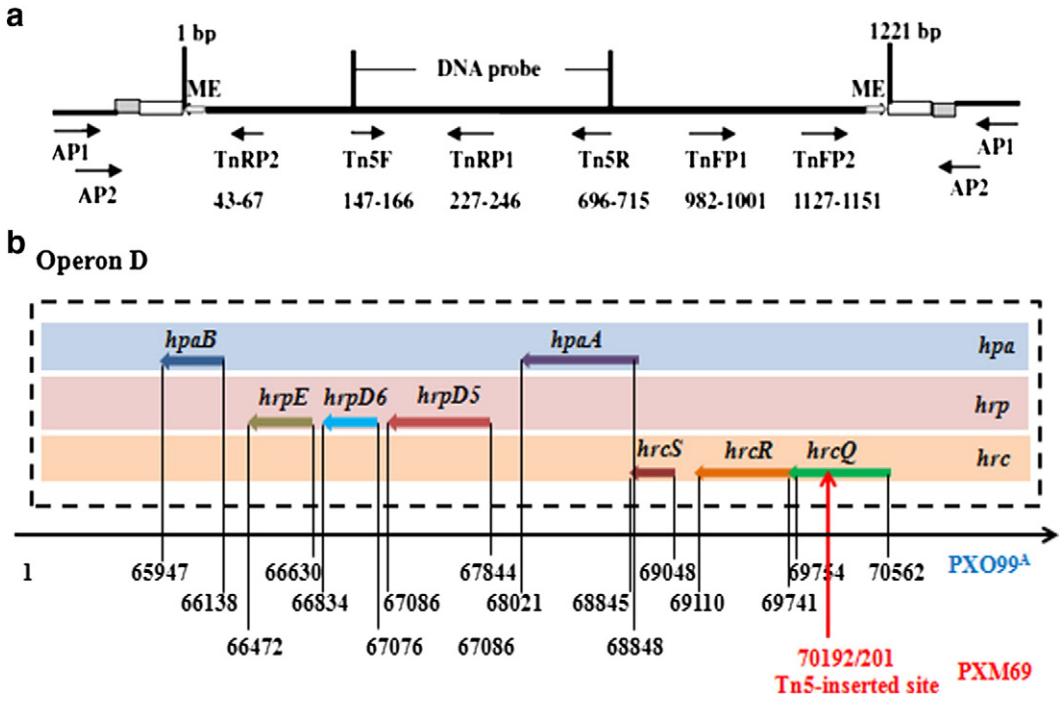

Fig. 3 – Tn5-insertion site in PXM69. a: Schematic representation of the Tn5-DNA insertion in the genome of PXM69. The 1221 bp Tn5-DNA is shown by the black bar with white-box arrows indicating the mosaic ends (ME). White bars represent the flanking sequences of *Xoo*. The adaptor used to isolate the flanking sequences is marked by "⎯". Black arrows under the bars represent the primers used in this study. Numbers under primer names correspond to the nucleotide positions of the primers. The position of the probe used for Southern blotting analysis is indicated. b: Schematic representation of the insertion site in the hrpD operon. Dashed box indicates the hrpD operon, *hpaA* is *hpa* (*hrp*-associated) gene, *hrpD5* and *hrpD6* are *hrp* (hypersensitive response and pathogenicity) genes, and *hrcQ*, *hrcR* and *hrcS* are *hrc* (*hrp*-conserved) genes. The long black arrow represents the genome of PXO99^{A}, under which the numbers indicate the nucleotide positions in the genome, including the Tn5-insertion site indicated in red.

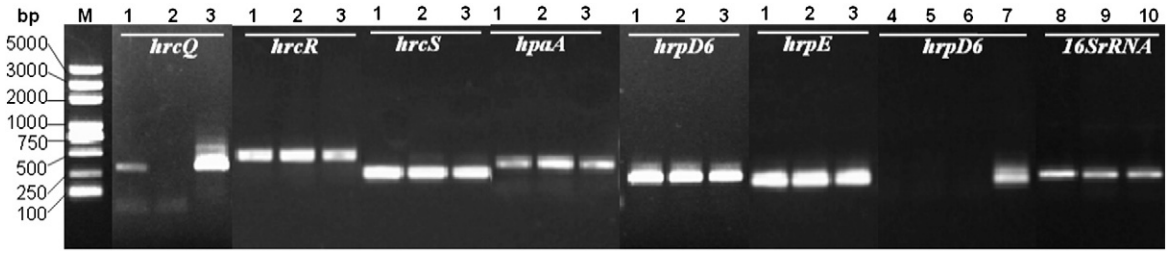

Fig. 4 – Reverse-transcription polymerase chain reaction (RT-PCR) analysis on the expression of *hrcQ* and the downstream genes in the D operon of *Xoo*. M: DNA molecular ladder with sizes (bp) as indicated. 1: PXO99^{A}; 2: PXM69; 3: pH-PhrcQ; 4–6: CK(–), RT-PCR using the RNA of PXO99^{A}, PXM69, and pH-PhrcQ, respectively, as template. Lack of amplification indicated that there was no DNA contamination in the RNA samples; 7: CK(+), RT-PCR using the DNA of PXO99^{A} as template; 8–10: RT-PCR of *16SrRNA* gene of the pathogen as the internal control to verify the absence of significant variation at the cDNA level in samples of PXO99^{A}, PXM69 and pH-PhrcQ as template. For the *hrcQ* and *16SrRNA* genes, RT-PCR was performed with 40 and 25 cycles, respectively. Others were performed with 35 cycles.

4. Discussion

In the present study, we identified and investigated the Tn5-insertion mutant PXM69 that had completely lost virulence in *indica* rice JG30. It was shown that a single Tn5 transposon inserted in the *hrcQ* gene within the *hrpD* operon of *Xoo* led to the loss of virulence in the rice host and of ability to elicit HR in non-host tobacco. This was confirmed by the recreated Δ*hrcQ*::KAN mutant of PXO99^{A}. However, reintroduction of the wild-type *hrcQ* gene into PXM69 did not completely complement the loss of pathogenicity. Since the 1326 bp *hrcQ*-containing DNA fragment (CP000967.1: 69,569–70,894) used for the complementation experiment contains the 822 bp *hrcQ* gene (CP000967.1: 69,741–70,562) and its promoter, and the sequence as confirmed by sequencing, some other factor(s) presumably affected the recovery of full pathogenicity of PXM69.

The clustered *hrp* genes are highly conserved in *Xanthomonas* [16]. The *hrpD* operon in *Xoo* contains eight genes from *hrcQ* to *hpaB* (*hrcQ-hrcR-hrcS-hpaA-hrpD5-hrpD6-hrpE-hpaB*) [9]. Recently, HrcQ has been demonstrated to be a core component of the T3SS in *Xoc*, facilitating Hpa1 and HrpB2 secretion through the T3SS to confer HR in tobacco and pathogenicity in rice [17]. Additionally, HrpD6 plays an important role in regulation of *hrp* genes and in the secretion of TAL effectors in *Xoc* [10]. In this context, the failure of complete complementation of PXM69 with wild-type *hrcQ* could not be explained. Since RT-PCR results showed that the expression of the downstream genes in the D operon was transcriptionally normal in mutant PXM69 and the complementary strain pH-PhrcQ (Fig. 4), the Tn5-insertion in *hrcQ* might affect the translation of proteins encoded by downstream genes in the D operon. This is worthy of verification in the future.

It is well known that pathogenicity of *Xoo* is determined by multiple genes. We isolated four PXO99^{A}-Tn5-insertion mutants with stably reduced pathogenicity in host rice JG30. Further investigation on the other three mutants may reveal other genes involved in the pathogenicity of *Xoo*.

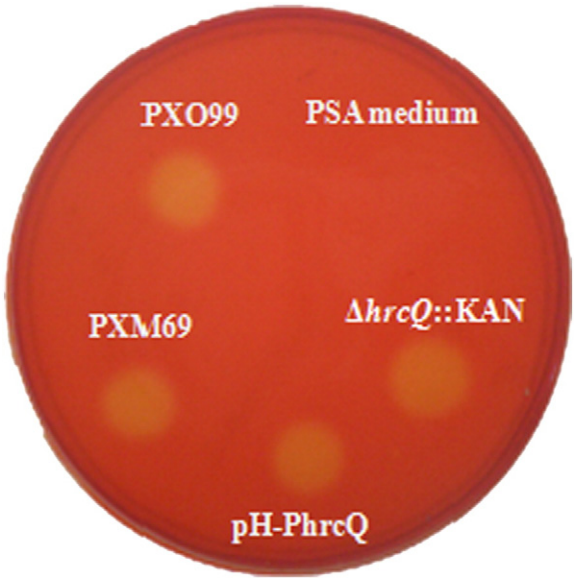

Fig. 5 – Cellulase activity assay of *Xoo* mutants. Cells of *Xoo* were spot inoculated on a CMC selection medium plate. Similar transparent halos were detected around colonies of PXO99^{A} (wild-type strain), PXM69 (Tn5-insertion mutant), Δ*hrcQ*::KAN (*hrcQ* gene disrupted by the kanamycin-encoding gene cassette), and pH-PhrcQ (complementary strain of PXM69). PSA medium was used as a negative control.

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